I*n vivo* and *in silico* evolution experiments highlight signatures of "evolution of evolution"

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Abstract. Evolution experiments, where living or digital ancestral organisms are propagated in specific environments, are exquisite tools to examine evolutionary mechanisms sustaining long-term adaption. Investigation of these *in vivo* and *in silico* fossil archives provide multiple evidence for the evolution of the evolutionary processes themselves as a key determinant of adaptive evolution.

Keywords: Evolution experiments, bacteria, digital organisms, mutation rates, regulatory networks.

All living organisms on Earth have been shaped through Darwinian evolution, *i.e.* through selection of random variants that are adapted to their contemporary fitness landscape. In the microbial world, evolution processes resulted in the extraordinary diversity of microorganisms and largely contributed to their ability to cope with both diverse and fluctuating environments. Indeed, microorganisms are organized as highly evolvable living systems with a genotype-to-phenotype map exquisitely fitting their environment. During the past decades, these properties inspired the development of experiments that were designed to reproduce microbial evolution either *in vivo* in the laboratory using living organisms [9, 16] or *in silico* in a computer using digital organisms [1, 2]. During such experiments, ancestral organisms are evolved for up to tens of thousands of generations (even more for digital organisms) in controlled environments and with systematic storage of both the ancestor and evolutionary intermediates, thereby providing a living and complete fossil record. Evolution experiments allow to dissect ecological, physiological, cellular and molecular mechanisms sustaining long-term adaptation (Fig. 1). Here, we will discuss how both *in vivo* and *in silico* approaches highlighted evolution of evolution (EvoEvo) as a major driver of long-term microbial adaptation.

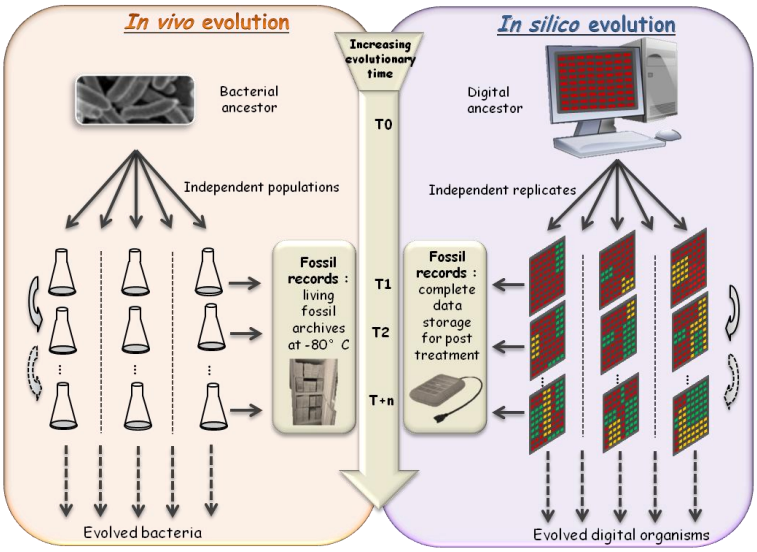

Fig. 1. *In vivo* and *in silico* experimental evolution. Ancestral microbial (left) or digital (right) organisms are propagated in controlled environments. The main advantage in these experiments is the availability of an ancestor and the evolved populations that are sampled throughout evolution. All living and digital organisms can be frozen or stored in databases, respectively, and revived at any time for further analyses.

Microorganisms and specifically bacteria constitute optimal ancestors for *in vivo* experimental evolution, since they have short generation times and large population size, and are easy to grow in the laboratory. The *Escherichia coli* bacterium, one of the most studied and characterized organism, is ideal to explore evolutionary processes and has been extensively used in many evolution experiments, since many tools are available to analyze genomes, global gene expression profiles and metabolic networks [11]. The longest-on-going evolution experiment, called the "long-term evolution experiment" (LTEE), was initiated in 1988 [16], and consists in twelve independent populations that are propagated from an *E. coli* B ancestral strain by daily serial transfers in a minimal glucose medium. In this experiment, bacterial populations thus evolve since now more than 60,000 generations and representative samples of each population were stored at -80°C at 500-generation intervals, thereby providing an unprecedented revivable fossil record. All twelve populations achieved large fitness gains [28] with several phenotypic traits evolving in parallel in most or all populations, including cell size, growth parameters, catabolic functions, and global gene expression [21]. Many other evolution experiments have been initiated using different ancestral organisms and different environmental settings [11, 13]. Here, we will discuss only about the LTEE since it is the model experiment used in the EvoEvo project. However, similar concepts and data have been discovered in other evolution experiments both with other microbial strains and environments. *In silico* evolution experiments are designed in a similar way with digital organisms instead of bacteria (Fig. 1). Digital organisms possess a genetic material that is interpreted by specific programs to compute the phenotype, and these simulated organisms compete, reproduce and mutate inside the computer. As for *in vivo* evolution experiments, all events are recorded and stored allowing retracing all the evolutionary intermediates during adaptation. Various frameworks have been developed to design digital organisms [11], and some are largely inspired from the genome organization of bacterial cells [2]. Besides the inherent limitation of such approaches using simulated, and thus simplified organisms, *in silico* evolution experiments allow for "perfect" experiments where all parameters (organismal traits, mutation rates, mutational bias, selection strength …) can be controlled, and with both the duration and replication level of the experiment only limited by the computational load.

The objective here is to highlight that, during the LTEE, many evolved changes reflect evolution of the evolutionary mechanisms themselves. Indeed, the evolutionary trajectories revealed a high dynamics of mutation rates, chromosomal structure, and global regulatory network architecture. In each of these cases, *in silico* models can be used as an exquisite complement to *in vivo* experiments to allow a more complete understanding of the underlying constraints.

1 Dynamics of mutation rates

During the LTEE, half of the twelve populations developed a hypermutator phenotype after mutations altering DNA repair systems [25, 27]. Because most mutations are deleterious, mutator alleles are likely to negatively impact fitness on average but theoretical work showed that they may reach fixation by hitchhiking with other, highly beneficial, mutations [26]. When hypermutable genotypes are established, they may contribute to rapid adaptation to environmental changes owing to their higher probability of producing rare beneficial mutations. However, hypermutators are also more likely to produce offspring carrying deleterious mutations, *i.e.* the genetic load is increased. This results in a tension between evolvability and stability that is likely to influence genomic evolution. Indeed, a high dynamics of mutation rates was recently identified in one population of the LTEE. This population first evolved into a hypermutator after the substitution of a *mutT* mutation resulting in a 150-fold increase in the mutation rate [27]. Later during evolution, when the population was adapted to its environment and therefore when the potential for further adaptation declined, two independent compensations of the high mutation rates occurred, resulting in two sub-lineages with reduced mutation rates [27]. It was further shown that this subsequent mutation rate decreases were benefi-

cial owing to the reduction of the genetic load. Such dynamics of mutation rates have been also observed in other evolution experiments [18, 20]. *In silico* evolution experiments have also shown that mutator alleles can promote accelerated adaptation [26]. Moreover, a theoretically optimal mutation rate has been computed using digital organisms with gradual evolution of mutation rates [3], although this value depends on the structure of the fitness landscape [4].

2 Evolution of the structure of the chromosome

Adaptation of the twelve bacterial populations of the LTEE is associated with many large chromosomal rearrangements, including inversions of more than one third of the chromosome, deletions and duplications with most events being mediated by IS elements [23]. Owing to their large size, these rearrangements are likely to strongly influence gene order, genome architecture and dosage of regulatory proteins within networks, thus potentially affecting growth traits and global transcription profiles. Some of these rearrangements, owing to their high level of parallelism across independent populations, have been suggested to be beneficial in these conditions. However, their precise impact on fitness has not been experimentally characterized, leaving open the question about the selective pressure that allowed the fixation of such events. (Large chromosomal rearrangements have been detected in other evolution experiments [8, 24]). In this context, *in silico* evolution experiments, in which direct selective pressures were precisely controlled and indirect pressures were less masked, are instructive because they have shown that indirect selection of evolvability can shape the genome structure at the levels of gene number, genome size, amount of non-coding DNA [15], and gene order [6].

3 Evolutionary rewiring of the global regulatory networks

During the LTEE, almost half of the characterized beneficial mutations affect genes that encode global regulatory proteins [21]. These mutational changes produce pervasive pleiotropic and epistatic effects [5], sometimes resulting in phenotypic innovation [22]. Therefore, long-term adaptation in this environment was achieved by substantial rewiring of global regulatory networks [Lamrabet et al., unpublished data], rather than by fine-tuning local regulators or structural enzymes. Regulatory networks therefore provide both immediate adaptation to environmental challenges by the flexibility of the regulatory connections and long-term adaptation by mutational changes of global regulator-encoding genes. Changes in regulatory networks are a hallmark of evolution experiments [17, 19, 29]. *In silico* evolution experiments also showed that regulatory networks are highly evolvable structures and provide powerful methods to generalize the observation made during *in vivo* evolution experiments. In particular, modularity of the networks has been investigated as a function of the environment [12] and in relationship with the evolvability and robustness of the organisms. Moreover, it has been shown that under fluctuating environments, regulatory networks can evolve toward exquisitely evolved structures [7].

4 Conclusion

In vivo and *in silico* evolution experiments separately provided ample examples that both microbial and digital organisms are able to evolve by modifying their evolutionary mechanisms themselves (mutation rates, genome structure and expression, cellular networks). However, we are far away from being able to suggest general rules and predictive evolutionary models. Collaborative and combined *in vivo* and *in silico* evolution experiments are urgently needed to address these issues, for instance to measure the impact of chance and history in the evolution of genomes and regulatory networks, and to identify generic properties that fuel evolutionary pathways. Indeed, *in silico* predictions not only have to be experimentally confirmed using *in vivo* experiments but will also target new *in vivo* experiments susceptible to demonstrate major determinants constraining evolutionary path-

ways. The EvoEvo project lies precisely at the frontier of *in vivo* and *in silico* evolution and combines state-of-the-art and complementary evolution models.

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